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**14. ABSTRACT**

The objective of this study was to identify and characterize biochemical markers of penetrating ballistic brain injury (PBBI). Candidate biomarkers were examined in a rat model for PBBI, with the goal of producing a panel of diagnostic and therapeutic-tracking biomarkers for penetrating brain injury. ELISA assays were developed and optimized to validate the biomarkers for PBBI. To assess the relationship between injury magnitude and biomarker levels, PBBI-treated rats were generated at three discreet injury magnitudes (5% moderate, 10% severe, 12.5% delayed lethal), plus controls. Levels of four biomarkers (SBDP150, SPDP145, UCHL1, GFAP) were measured in collected biofluids and/or cortex, using our ELISAs and immunoblotting. We found that SBDP150 levels were significantly elevated at early times after PBBI in CSF and blood-derived plasma. UCHL1 was also elevated in early plasma samples, making SBDP150 and UCHL1 candidates for diagnosing severe PBBI in acute settings from a blood sample. Levels of all four biomarkers showed stepwise increases in biofluids and/or cortex as injury magnitude increased. Additionally, GFAP levels in the CSF were lowered by treatment of PBBI animals with drugs, indicating that GFAP is a useful biomarker for monitoring progress after therapeutic interventions. Overall, our results robustly support the conclusion that biomarkers are effective indicators of PBBI that can track injury magnitude and therapeutic progress. Results are reported here in full.

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## INTRODUCTION:

The purpose of this work was to develop means of assessing the severity of brain injury produced primarily by bullet or shrapnel wounds to the head by means of simple blood tests that could be easily conducted even by a medic in a combat environment. This research was guided by the general idea that specific proteins or their fragments detectible in CSF or blood are biochemical markers capable of accurately diagnosing penetrating ballistic brain injury (PBBI). Critically, use of biomarker-based diagnostics in combat environments will enable rapid medical triage assessments to direct medical strategies and/or evacuation priorities. Also, accurate diagnosis in acute care environments can significantly enhance decisions about patient management, including decisions whether to admit or discharge or administer other time consuming and expensive tests. Ideally, biomarkers should employ readily accessible biological material such as CSF or blood (CSF is accessible in severely injured PBBI patients), predict the magnitude of injury and resulting functional deficits, possess high sensitivity and specificity, have a rapid appearance in CSF and/or blood, and be released in a time-locked sequence after injury (Mondello, et al. 2010a). Once candidate biomarkers were identified, biomarker validation was performed using quantitative immunoblotting and ELISA methods. Finally, the ability of identified biomarkers to track the progress of pharmacological therapy was evaluated.

**BODY:** The specific aims, overall, were not altered.

*SOW 1: will employ global, high throughput proteomic and metabonomic technologies, as well as targeted approaches, to identify proteins, peptide fragments or metabolites that change in abundance as a result of PBBI and are potentially released into CSF, blood of rats following experimental PBBI (SOW 1A). We will also determine if previously identified injury-related proteins or peptide fragments and associated biomarkers of other forms of acute brain injury are also potential biomarkers of PBBI (SOW 1B). A metabonomic analysis for biomarker discovery in urine is also underway to process (SOWC).*

Rat cortex, CSF and plasma were generated from the following groups/time points: PBBI (10% severity level) at 3 time points (2h, 24h, 7d) vs. sham control (24h, 7d) and naïve, with 5 rats per group. CSF and serum samples were also collected for SOW2. We examined by immunoblotting several potential biomarkers including αII-Spectrin breakdown products (SBDPs) in cortex (Dutta, et al. 2002, Zhang, et al. 2009). Quantification of SBDPs and statistical analyses show that SBDP145 was significantly increased at 24h after PBBI in the cortex while SBPP120 was not significantly changed (**Fig. 1**). We also confirmed these results using fragment specific antibodies to SBDP145 and SBDP120 with β-Actin as loading control (**Fig. 2**). **Fig. 3** shows that glial fibrillary acidic protein (GFAP) (Lumpkins, et al. 2008) induction was dramatic on day 7 after PBBI in the cortex. This makes GFAP a candidate biomarker for detecting PBBI.

We have also found that Neural Cell Adhesion Molecule (NCAM) (Covault, et al. 1991), which may be a novel biomarker of TBI, had reduced levels of its isoforms at 140 kDa and 180 kDa in the cortex at 2h, 24h and 7d after PBBI compared with sham and naïve (**Fig. 4A,B**). Our results showed that NCAM is specifically expressed in brain **(Fig. 4C).** Our preliminary data have revealed that NCAM is digested by calpain, not caspase (**Fig. 5A**). We attempted to use N-terminal sequencing to identify the novel

cleavage site(s) in NCAM produced by calpain (**Fig. 5B**). However, after a change in personnel results could not be repeated, and therefore we decided not to pursue NCAM further at this time.

We had planned to use metabonomic analyses for biomarker discovery in urine following PBBI. We collected total 24 urine samples from 5 different groups which included naïve, and sham and PBBI at two time points each (1 day, 7 days). However, despite repeated attempts Banyan was unable to reach the personnel who had originally agreed to do the analyses. Therefore by necessity the funds for metabonomics were reallocated to other areas of active work.

*SOW 2: will evaluate potential biomarkers identified in SOW 1 for selection of the most promising candidates and develop sensitive assays capable of detecting these markers in complex biofluids. First, studies will independently confirm the detection of putative biomarkers from SOW 1 in CSF and serum by immunoblotting. Ultimately, optimal biomarkers will be identified (SOW 2A). We will then develop a minimum of 4-6 highly sensitive, quantitative ELISA based assays of optimal biomarkers identified in SOW 2A, which are capable of detecting biomarkers in CSF and/or blood (SOW 2B).*

We have developed, then performed sensitive, quantitative ELISA assays on the best biomarkers, SBDP145 kDa and GFAP (Pelinka, et al. 2004, Vos, et al. 2004, Pineda, et al. 2007{Lumpkins, 2008 #78, Brophy, et al. 2009, Mondello, et al. 2010b, Berger, et al. 2011, Mondello, et al. 2011b, Papa, et al. 2011). The ELISAs are capable of detecting these biomarkers in both CSF and blood. Preliminary data show that SBDP145 kDa significantly increased at 2h and 24h after PBBI in the CSF (**Fig. 6A**) and dramatically increased at 24h after PBBI in the serum of PBBI rats (**Fig. 6B**), which is consistent with the data from our Western blots on the cortex of PBBI rats. While GFAP inductions were seen be significantly changed at all the time points in the CSF (**Fig. 7A**), there is only a significant increment at 2h time point in the serum of PBBI rats (**Fig. 7B**). We will repeat the assay on serum samples with added numbers to confirm these results. Thus overall, our preliminary data suggested that αII-Spectrin SBDP145 kDa and GFAP significantly increase in CSF and plasma after PBBI at early time points, and thus that these may serve as biomarkers during acute or sub-acute phases of PBBI. We are further optimizing our in-house sandwich ELISA for GFAP to improve detection sensitivity. We also developed ELISAs for SBDP150 and UCHL1 (ubiquitin carboxy-terminal hydrolase-like 1), which are also used in SOW3 (Brophy, et al. 2011, Mondello, et al. 2011a). Therefore we have ELISAs for 4 biomarkers total to apply to PBBI samples.

*SOW 3: will employ ELISA based assays to monitor post-injury biomarker kinetics and answer how levels are affected by PBBI injury magnitude (moderate, severe, delayed lethal). For this, serial serum samples will be drawn at multiple time points (5min, 2hr, 6hr, 24hr, 3d, 7d). Biomarker levels will be analyzed, and correlated with neurological dysfunction. Lastly, the ELISA based assays will be employed to monitor changes in biomarker levels to track the effects of Dextromethorphan therapy and correlation to histopathology and neurological outcome.*

First we tested whether biomarker levels correlated with injury magnitude, utilizing the developed ELISA assays from SOW 2. We studied serial changes in blood-based biomarkers as a function of injury severity, using three discreet injury magnitudes based on the volume of brain that was injured, as defined by the percent of total brain injured: 1. Moderate, 5% PBBI: causing pathological damage and neurodysfunction, but 100% survivable; 2. Severe, 10% PBBI: causing pathological damage and neurodysfunction, but with 30% mortality; 3. Delayed lethal, 12.5% PBBI: causing 100% mortality but after a time delay (Williams, et al. 2005, Williams, et al. 2006).

For these biomarker studies, new sets of rat cortex, CSF and plasma were generated from five different groups of rats (naïve control, sham control, 5% PBBI, 10% PBBI, and 12.5% PBBI), with 5 rats per group. Blood for plasma was collected at 6 time points (5 min, 2 hr, 6 hr, 24 hr, 3 days, 7 days), and brain cortex and CSF were collected at two time points (1 day, 7 days). Levels of the 4 biomarkers (SBDP150, SBDP145, UCHL1, GFAP) were measured in biofluids at each time point using the ELISA assays, and/or in brain tissue using quantitative immunoblotting.

SBDP150 and SBDP145 are breakdown products of the structural protein αII-spectrin which are detectable in dying cells (Zhang, et al. 2009). In our studies reported here, we found that SBDP150 levels in CSF and plasma were significantly increased at early times post PBBI (**Figs 8,9**). In CSF, SBDP150 levels in severely injured animals at 10 and 12.5% PBBI were significantly elevated over sham 1 day after injury (**Fig. 8A**). In plasma, SBDP150 levels at the same injury magnitudes were significantly higher than controls from 5 minutes to 6 hours after injury (**Fig. 8B**). In cortex isolated from animals at 1 day post injury, SBDP150 levels closely correlated with injury magnitude. There was a clear step-wise increase in SBDP150 level as injury severity increased, with levels becoming significant at 10 and 12.5% PBBI (**Fig. 9A**). Similarly, levels of SBDP145 tracked injury magnitude very well at 1 day after injury, with all 3 degrees of injury showing a significant difference from sham (**Fig. 9B**). A representative blot used for quantifying SBDP levels is shown (**Fig. 9C**). Therefore αII-spectrin breakdown products showed good promise as biomarkers for PBBI, especially for severe injuries. For instance, the rapid appearance of SBDP150 in blood plasma could potentially make this a useful biomarker for diagnosing severe penetrating ballistic wounds on the battlefield, if combined with development of a rapid detection method.

UCHL1 is a deubiquitinating enzyme that is abundantly expressed in neurons, and which is associated with neurodegenerative diseases (Setsuie and Wada 2007). In our studies, we discovered that UCHL1 is significantly elevated in the CSF of the most severely injured rats compared to naïve animals at 1 day after injury (12.5% PBBI, **Fig. 10A**). It is also significantly increased in plasma 5 minutes after injury at 10 and 12.5% PBBI (**Fig. 10B**). Although not as robust as SBDP150, it could also be useful as a biomarker in severe PBBI patients.

GFAP is an abundant intermediate filament protein in central nervous system glial cells such as astrocytes that is correlated with brain injury (Lumpkins, et al. 2008). We found that GFAP was remarkably effective at tracking injury magnitude when measured in the CSF 1 day after injury (**Fig. 11**). GFAP levels at all 3 injury magnitudes were significantly higher than controls, either naïve (5%) or sham

(10 and 12.5%). Additionally, there was a significant upward increase in GFAP level between 5 and 10% PBBI, with a stepwise increasing trend in GFAP across all injury levels. We measured GFAP in plasma taken at the 6 serial time points as for the other markers, however the readings either failed or were very low, so no chart is shown and there is no data to report. We also measured levels of full length GFAP at 49kDa, and a smaller GFAP fragment at 45kDa in rat cortex by immunoblotting. We found that full length GFAP was significantly elevated at all injury magnitudes compared to either naïve or sham rats at 7 days (**Fig. 12A**). The sham-injured animals also had elevated GFAP levels in this experiment, suggesting that they were injured during the sham operation. We need to be more careful with this in the future. GFAP 45kDa was also significantly upregulated at 10 and 12.5% PBBI at 7 days (**Fig. 12B**). Interestingly, in contrast to SBDP150 which was elevated in both the CSF (**Fig. 8A**) and brain (**Fig. 9A**) 1 day after injury, GFAP was elevated in the CSF at the 1 day time point (**Fig. 11**), but in brain at the 7 day time point (**Fig. 12**). GFAP is thus unique in being elevated early in the CSF, and later in the injured brain. It is unclear why levels of GFAP in the CSF at 7 days did not reflect the increased levels in brain at this time point. Nevertheless, based on the data in Fig. 4, GFAP is a promising biomarker for early detection of PBBI when measured in the CSF.

Next we examined whether these biomarkers could be used effectively to track the progress of drug therapies for PBBI. All these experiments were all done with one injury magnitude (10% PBBI). First we tested a derivative of glycine-proline-glutamate (GPE). GPE is an N-terminal tripeptide endogenously cleaved from insulin-like growth factor-1 in the brain that is neuroprotective against hypoxic-ischemic brain injury and neurodegeneration. We used NNZ-2566 as a therapeutic agent, which is an analog of GPE designed to have improved bioavailability. Our collaborators, Dr. Tortella's group in WRAIR, have already demonstrated that NNZ-2566 improves functional recovery and attenuates apoptosis and inflammation in rat PBBI model (Lu, et al. 2009, Wei, et al. 2009). While GFAP was dramatically increased in CSF at 4h, 24h and 72h post-PBBI, NNZ-2566 treatment significantly reduced GFAP in CSF at each time point post-PBBI **(Fig. 13A)**. Similar results were seen in the plasma 4h post-TBI (**Fig. 13B**). Therefore this preliminary analysis supports the use of biomarkers for therapeutic tracking.

Next we tested another therapeutic agent for PBBI, dextramethorphan (DM). DM is an antitussive known to have positive effects on outcome after PBBI in rats (Shear, et al. 2009). For these experiments, we generated 5 groups of 10 rats each (naïve, sham, vehicle, DM low dose 0.156mg/kg, DM high dose 10mg/kg). Sham animals were subjected to craniotomy but not injured, while vehicle and DM rats were exposed to 10% PBBI. CSF, blood, and cortex were collected 24 hours post injury. Biomarkers (SBDP150, SBDP145, UCHL1 and GFAP) were measured in brain and biofluids by quantitative ELISA and/or immunoblotting. SBDP150 levels were not lowered in the CSF or plasma of PBBI rats treated with DM compared to vehicle control (**Fig. 14A,B**). SBDP150 and SBDP145 levels showed downward trends in brain with DM treatment, although the changes did not reach statistical significance (**Fig. 14C,D**). UCHL1 did not change with DM treatment in CSF or plasma (**Fig. 14E,F**). Notably, while GFAP levels were significantly increased by 10% PBBI, levels did not differ from sham in PBBI animals treated with DM (**Fig. 14G**). Importantly, this result suggests that GFAP levels returned to baseline after DM, and supports GFAP as a biomarker for monitoring pharmacological therapy. GFAP levels in the cortex did not change with DM (**Fig. 14H**).

## METHODS:

**Animal Usage:** All PBBI injuries were produced in male Sprague-Dawley rats (250-300 g; Charles River Labs, Raleigh, VA). All procedures were approved by the Walter Reed Army Institute of Research Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals. Animals were housed individually under a 12 hour light/dark cycle (lights on at 0600) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All surgical procedures were performed on anesthetized animals. Anesthesia was induced and maintained with 2-5% isofluorane delivered in oxygen. Body temperature was maintained normothermically (37 ± 1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Following surgery, animals were placed in a warm chamber maintained by a circulating water-bath heating system (Gaymar Indust., Orchard Park, NY) until recovery from anesthesia. Food and water were provided ad libitum post-operatively.

**Unilateral frontal PBBI Injury:** A PBBI apparatus (MITRE Corp., McClean, VA.) was used to simulate a ballistic injury of a high-velocity 7.62 mm NATO round to the rat brain [Williams, 2005]. Briefly, rats were anesthetized and positioned in a stereotaxic frame for probe insertion. The scalp was incised along the midline and a cranial window (4 mm in diameter) was created to expose the right frontal pole of the brain. The PBBI probe was made of a 20G stainless steel tube with fixed perforations along one end and sealed by a 1 cm piece of elastic tubing. The probe was secured on the probe holder attached to the stereotaxic frame at an angle of 50 degrees from the vertical axis and 25 degrees counter clockwise from the midline. The probe was advanced manually along this axis through the cranial window into the brain. One end of the probe was connected to a hydraulic pressure generator. Upon activation by a computer, the generator rapidly (< 40ms) inflated and deflated the elastic tubing on the probe to an elliptical-shaped water balloon. The magnitude of PBBI was controlled by inflating/deflating the PBBI balloon to a diameter equal to 0.48 cm, 0.63 cm or 0.70 cm, which represents 5, 10 or 12.5% of total rat brain volume, therefore defining three PBBI severity levels. Immediately following deflation, the probe was gently retracted from the brain, the cranial opening sealed with sterile bone wax, and the incision closed with 4-0 nylon suture, followed by administration of a topical antibiotic. Sham animals were not subjected to probe insertion but otherwise received all surgical manipulations. Serial blood samples were collected via indwelling right jugular vein catheter. For the dextramethorphan study, vehicle (saline) or DM (0.156 or 10 mg/kg, Sigma-Aldrich) was delivered as i.v. bolus injections at 30 min post-injury (n=10 per group), and animals were sacrificed 24 hr later (Shear, 2009).

**Quantitative Immunoblotting analysis**: GFAP bands were counted individually and then summed to yield the total amount of GFAP protein. Primary antibodies used were mouse anti-αII-spectrin, GFAP, β-actin, and Cyclophillin A. Semi-quantitative evaluation of protein and BDP levels was performed via computer-assisted densitometric scanning (Epson XL3500 high resolution flatbed scanner) followed by image analysis with Image-J software. The amount each biomarker is displayed in arbitrary densitometric units.

**Biomarker sandwich ELISA assays**: CSF and plasma samples were analyzed with Banyan Biomarker's proprietary sandwich enzyme-linked immunosorbent assays (SW-ELISA) for UCHL1, SBDP150 and SBDP145. Selected capture and detection antibody pairs were used. Briefly, ELISA plates (NUNC Maxisorp high binding) were passively coated overnight at 4C with capture antibody, washed and blocked for 30 minutes. CSF (5-10 µl) or plasma (20-34ul) samples were incubated for 2 hours at room temperature with shaking. After washing, detection antibody working solution was added and incubated for 90 minutes with shaking. Peroxidase-conjugated detection antibody or streptavidin-HRP conjugates catalyzed the reaction with a colorimetric substrate (TMB) and product was quantitated by absorbance at 450 nm in a microplate spectrophotometer. For GFAP in CSF, 6.8ul per sample was measured using a commercial ELISA kit (QC-SL-001, BioVendor) according to the manufacturer's instructions. Standard curves were generated using recombinant proteins corresponding to the biomarker measured in each assay. Four parameter-fit non-linear regression analyses were applied to determine biomarker quantities. For simplicity, only sham controls are shown in plasma timecourse charts.

**Statistical analyses:** Primary quantitative data from immunoblotting and ELISA assays were compiled and charted in Excel, and statistical analysis were applied using GraphPad Prism 5.0. Occasional outliers were excluded. Two way ANOVA (Analysis of Variance) was applied to all datasets as the test for statistical significance. Two way ANOVA was followed by the Bonferroni post test to determine p values for differences between groups within each experiment. In all cases, $p<0.05$ was the criterion for significance. Analyses for CSF and cortical biomarker levels were derived from five animals per group. For plasma measurements, there were 10 rats per group for the 1 day time point, and 5 rats per group for all other time points. For the dextramethorphan study, there were 10 animals per group.

## KEY RESEARCH ACCOMPLISHMENTS:

-Identified NCAM as a calpain target suggesting NCAM-BDP as a potential biomarker for PBBI.

-Developed quantitative ELISA assays for four biomarkers and demonstrated the effectiveness of these assays for measuring these biomarkers in biofluids.

-Demonstrated the elevation of αII-spectrin SBDP150 in cortex, CSF and plasma after PBBI in rats, and its correlation with injury magnitude in cortex and CSF.

-Demonstrated the elevation of SBDP145 in cortex post PBBI, and its correlation with injury magnitude.

-Demonstrated the elevation of GFAP in brain at 7 days and CSF at 1 day after PBBI in rats, and its correlation with injury magnitude in CSF.

-Demonstrated the elevation of UCHL1 in CSF and plasma after PBBI in rats, and its potential correlation with injury magnitude in CSF.

-Showed that GFAP levels in biofluids respond to two therapeutic drugs for PBBI, indicating GFAP is useful for tracking therapy in PBBI animals.

## REPORTABLE OUTCOMES:

-A poster was presented at the ATACCC conference in Orlando, Florida, 2009.

-An oral report was presented at the Military Health Research Forum in 2009.

-A poster was presented at the National Neurotrauma Society meeting in Fort Lauderdale, Florida, 2011.

-A manuscript describing our results is being prepared and its planned submission date is January 2012, entitled, "BIOMARKERS TRACK INJURY SEVERITY IN A RAT MODEL OF PENETRATING BALLISTIC BRAIN INJURY," by J. Susie Zoltewicz et al.

## CONCLUSION:

Our studies have contributed to the field of PBBI by demonstrating that the αII-Spectrin breakdown products SBDP150 and SBDP145 kDa, as well as UCHL1 and GFAP are promising biomarkers for PBBI. We also identified NCAM as a brain-specific protein that is degraded by calpain. NCAM was found to decrease after PBBI and deserves further study, provided our preliminary results can be repeated. Because GFAP increases occurred earlier in CSF than the cortex, GFAP may serve as a predictor of injury or gliosis. Moreover, with our studies we have met an important and primary goal, which was to identify one or more biomarkers in accessible biofluids that could track the severity of PBBI. GFAP met this criteria, as its concentration in CSF was significantly increased between moderate (5% PBBI) and severe (10% PBBI) injury magnitudes. There was an additional increase in GFAP CSF levels between 10% and 12.5% PBBI which is obvious but not statistically significant, but the sample size used was small (5 animals per group), strongly suggesting that it would become significant in a larger group. Similarly, SBDP150 (5-10%) and UCHL1 (5-12.5%) showed stepwise upward increases in CSF as injury magnitude increased, again suggesting that these increases would become significant if sample size is increased. Given their early detection in plasma, SBDP150 and UCHL1 could potentially be used to diagnose severe PBBI on the battlefield or soon after injury occurs, from a blood sample. Therefore we have promising biomarkers for early detection of PBBI and for tracking injury magnitude. In addition, our results suggested a correlation between the level of GFAP in biofluids and NNZ-2566 therapy post-PBBI. Likewise, the level of GFAP released into CSF after PBBI was reduced by DM therapy, indicating that GFAP is a useful biomarker for tracking progress after therapeutic interventions. Overall, our results robustly support the conclusion that biomarkers are effective indicators of ballistic brain injury. We are therefore pleased to report that we have generated a full set of exciting data to support our hypotheses and stated goals. A manuscript presenting our best data and representing the culmination of this grant has been prepared by J. Susie Zoltewicz, Ph.D. We plan to submit this manuscript for publication in January 2012.

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## **APPENDIX**:

A manuscript describing our results will be submitted as an appendix to this report, with title and authors as follows:

**BIOMARKERS TRACK INJURY SEVERITY IN A RAT MODEL OF PENETRATING BALLISTIC BRAIN INJURY**

J. Susie Zoltewicz, Boxuan Yang, Kimberly Newsom, Changping Yao, Xi-Chun May Lu, Jitendra R. Dave, Deborah A. Shear, Virginia Rivera, Terri Cram, Jixiang Mo, Richard Rubenstein, Kara Schmid, Frank C. Tortella, Ronald L. Hayes, Zhiqun Zhang and Kevin K. W. Wang

## SUPPORTING DATA:

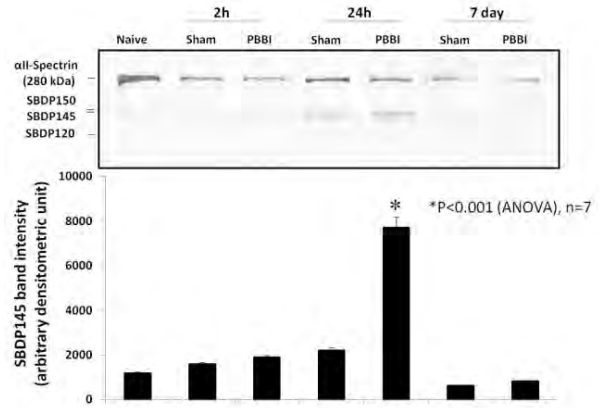



**Figure 1. Quantitative immunoblotting shows that SBDP145 was increased 24h after 10% PBBI in cortical tissue.** **A.** Representative blot is shown. **B**. SBDP145 levels are shown based on densitometric quantitation, and ANOVA analysis was performed to determine significance.

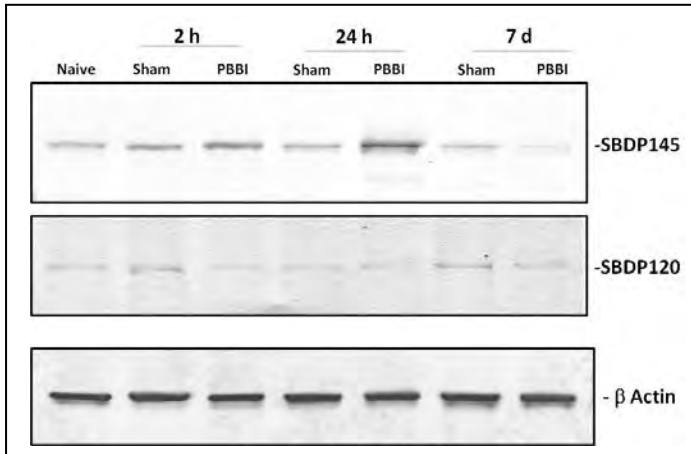



**Figure 2. Fragment-specific antibodies confirm elevation of SBDP145 but not SBDP120 after 10% PBBI in cortex.** Representative blots are shown. Actin served as the loading control.

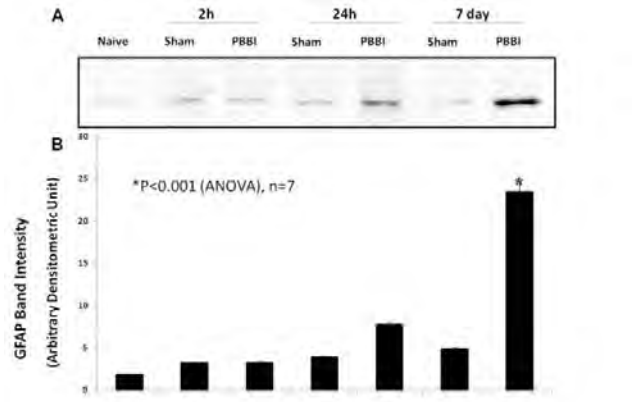



**Figure 3. Quantitative immunoblotting shows that GFAP was robustly elevated 7 days after 10% PBBI in cortex.** **A.** Representative blot is shown. **B**. GFAP levels are shown based on densitometric quantitation, and ANOVA analysis was performed to determine significance.

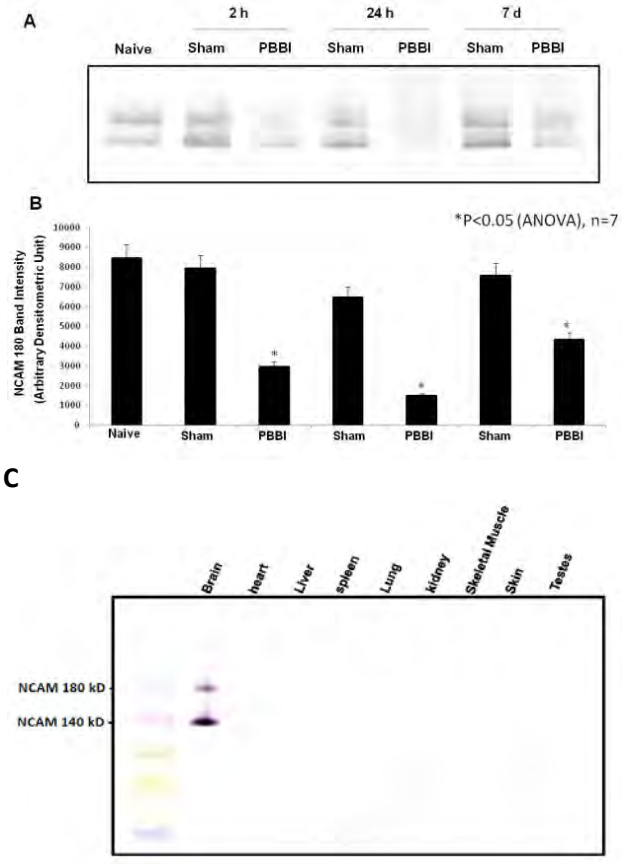
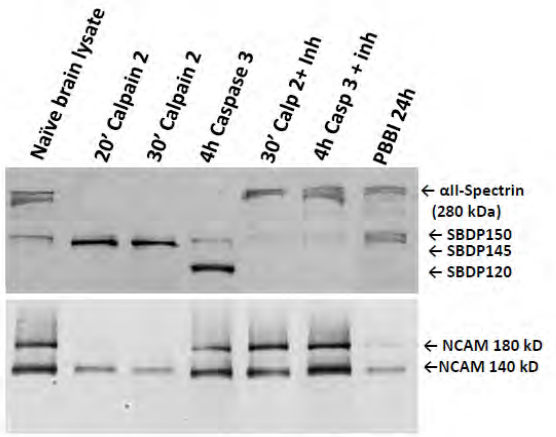



**Figure 4. NCAM, a brain specific protein, was reduced after 10% PBBI in cortex.** **A.** Representative blot is shown. **B.** The 180kDa fragment is quantified based on densitometry. **C**. In normal tissues, NCAM is brain-specific. ANOVA analysis was performed to determine significance.

**A.**




**B.**

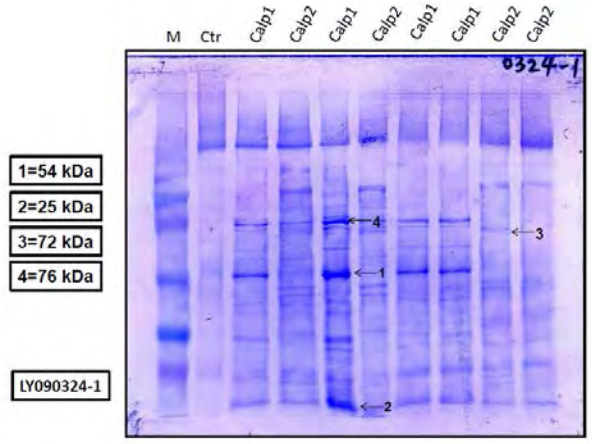
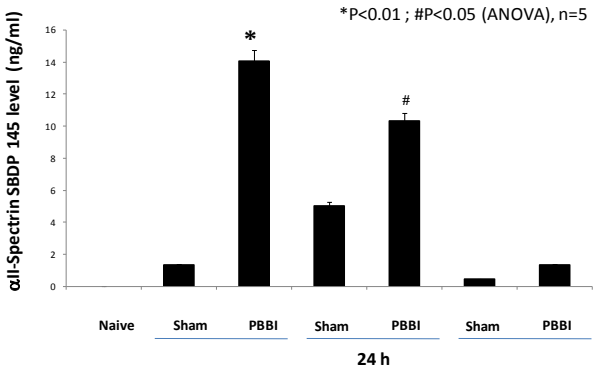



**Figure 5. NCAM is a calpain target.** **A.** NCAM was degraded by calpain, not caspase. **B.** Degraded NCAM bands were stained with Coomassie and the bands indicated at the left were cut out for N-terminal sequencing.

**A. SBDP145 in CSF**




**B. SBDP145 in serum**

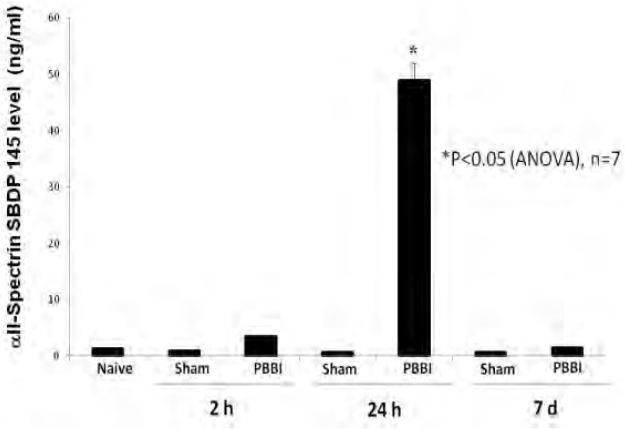
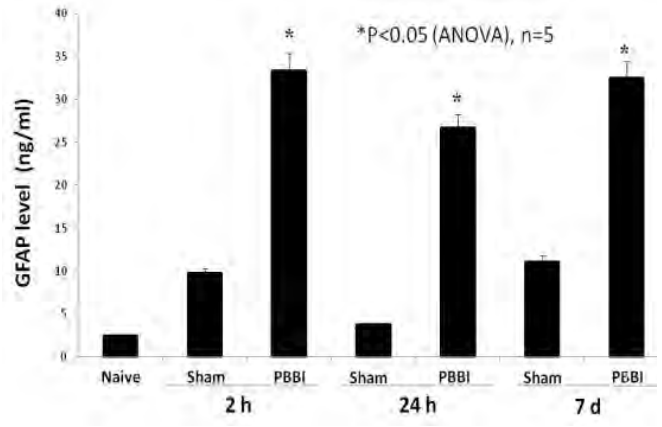



**Figure 6. SBDP145 was increased in CSF and serum after 10% PBBI (ELISA).** **A.** SBDP145 was significantly elevated in the CSF at 2h (left) and 24h (middle) after injury. **B.** SBDP145 was robustly increased in serum 24h after injury. ANOVA analysis was performed to determine significance.

**A. GFAP in CSF**




**B. GFAP in serum**

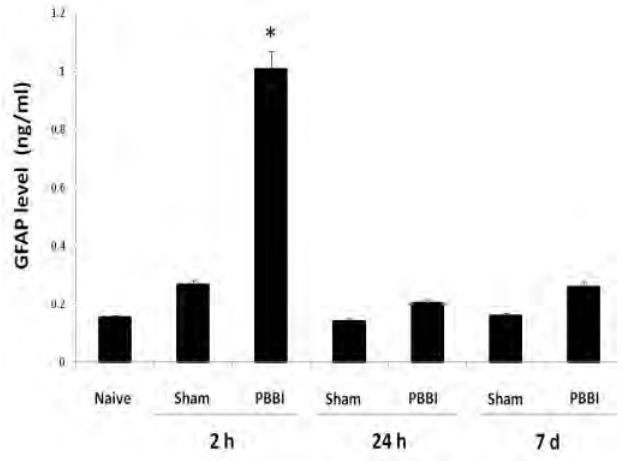



**Figure 7. GFAP was increased in CSF and serum after 10% PBBI (ELISA).** A. GFAP in CSF rose at all timepoints. B. GFAP in serum was elevated 2 hours after injury ANOVA analysis was performed to determine significance.

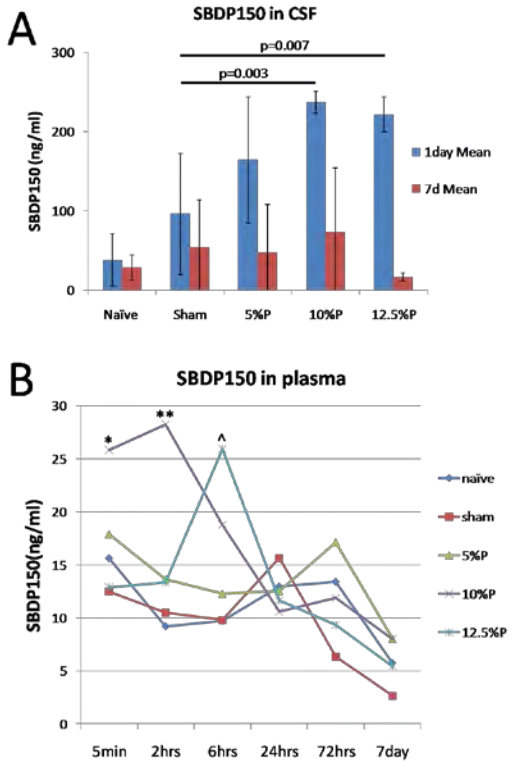



**Figure 8. The αII-spectrin breakdown product SBDP150 is significantly elevated in the CSF and plasma of animals at early times post PBBI.** SBDP150 levels were quantified by sandwich ELISA. The mean levels of SBDP150 in the indicated biofluid of 5 animals per group are plotted along with standard deviations. **A.** SBDP150 CSF levels in severely injured animals (10% and 12.5% PBBI) are significantly higher than sham and naïve 1 day post PBBI. p values are as shown. **B.** In animals with 10% PBBI (purple), SBDP150 plasma levels are significantly elevated 5 minutes and 2 hours post PBBI compared to sham (*p=0.017, **p=0.01). In animals with 12.5% PBBI (light blue), SBDP150 levels are significantly higher 6 hours post PBBI compared to sham-injured animals (^p=0.005). P values by ANOVA.

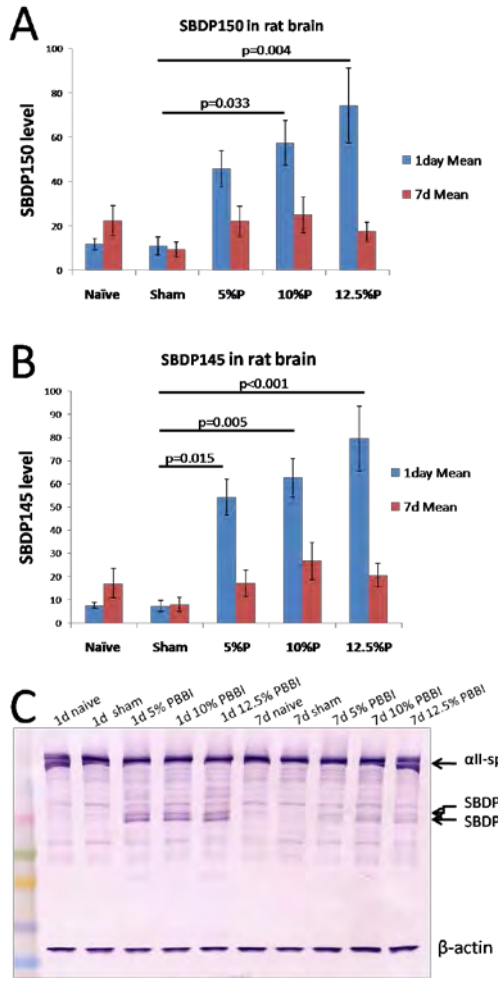



**Figure 9. Levels of the αII-spectrin breakdown products SBDP150 and SBDP145 in brain track injury magnitude at 1 day post PBBI.** The mean levels of SBDPs calculated from 5 animals per group are shown along with standard deviations. p values compared to sham injury are shown (ANOVA). **A.** Brain levels of SBDP150 in severely injured animals (10% and 12.5% PBBI) are significantly higher than sham and naïve. **B.** Levels of SBDP145 are significantly elevated at all 3 injury magnitudes compared to sham and naïve. **C.** SBDP levels shown in A and B were determined by quantitative immunoblotting and a representative blot of brain lysates is shown. Actin shows equal loading.

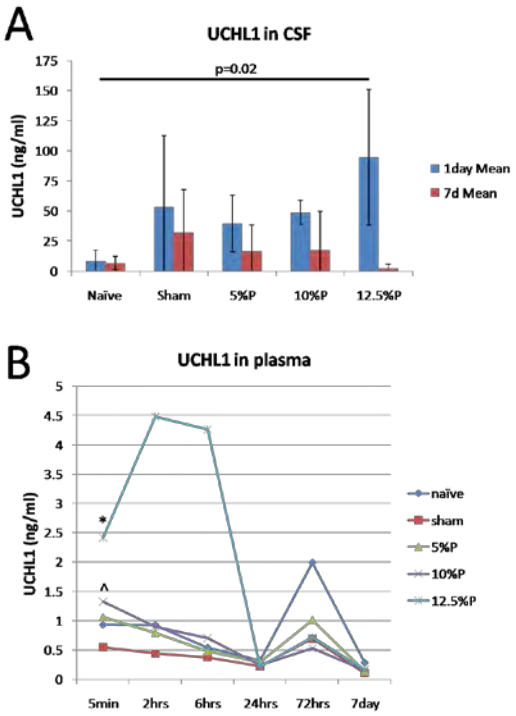



**Figure 10. UCHL1 is significantly elevated in the CSF and plasma of animals at early times post PBBI.** UCHL1 levels were quantified by ELISA. The mean levels of UCHL1 in the indicated biofluid of 5 animals per group are plotted and standard deviations are shown. **A**. UCHL1 CSF levels in the most severely injured animals (12.5% PBBI) are significantly higher than sham and naïve 1 day post PBBI. p value is shown (ANOVA). **B**. In animals with 10% PBBI (purple, ^p=0.002 ANOVA) and 12.5% PBBI (light blue, *p<0.001), UCHL1 plasma levels are significantly elevated 5 minutes post PBBI compared to sham (^p=0.002). Although 12.5% PBBI levels are also elevated at 2 and 6 hours post injury, these increases are not statistically significant due to high standard deviations.

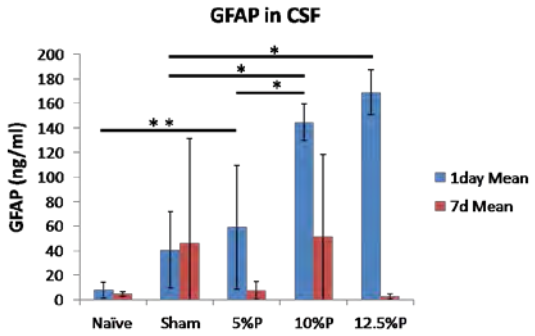



**Figure 11. GFAP in CSF tracks injury magnitude at 1 day post PBBI.** GFAP level increases with injury severity, and increases are highly significant at 10% and 12.5% PBBI. *p<0.001, also significant compared to naive; **p=0.043. Quantitated by ELISA, 5 animals per group.

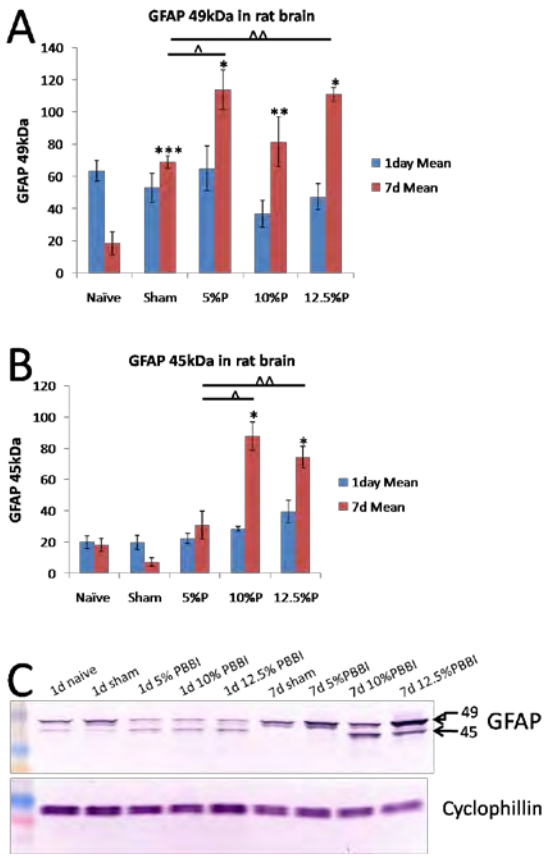
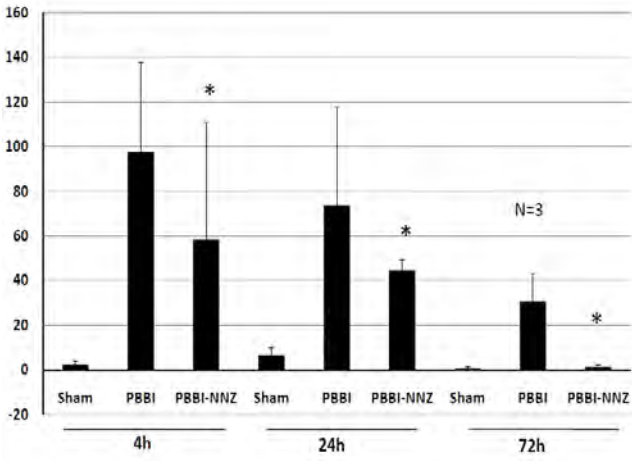



**Figure 12. GFAP is significantly increased in brain at all injury magnitudes 7 days post PBBI.** GFAP levels were quantified by ELISA, 5 rats per group. **A.** Full length GFAP at 49kDa is elevated in brains of PBBI animals. Asterix indicate significance compared to naïve animals at 7 days (*p<0.001, **p=0.001, ***p=0.01 ANOVA), and ^ indicate significance compared to sham-injured animals at 7 days (^p=0.023, ^^p=0.03). **B.** The 45kDa cleaved isoform of GFAP is increased several fold at 10% and 12.5% PBBI. Asterix indicate significance compared to naïve and sham at p<0.001 at 7 days, and ^ indicate significance compared to 5% PBBI with ^p<0.001 and ^^p=0.001 at 7 days. **C**. A representative quantitative immunoblot used to obtain the data plotted in A and B is shown. Cyclophillin served as a loading control.

**A. GFAP in CSF with NNZ**




**B. GFAP in plasma with NNZ**

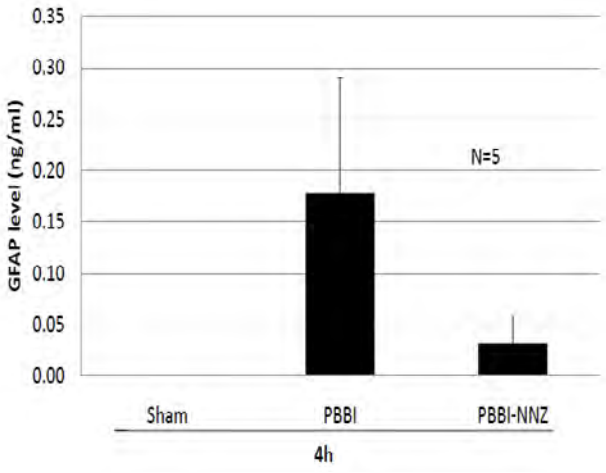



**Figure 13. GFAP was decreased in CSF (A) and plasma (B) after animals with 10% PBBI were treated with NNZ-2556. A.** GFAP in CSF is shown. **B.** GFAP in plasma is shown. GFAP levels were determined by ELISA. ANOVA analysis was performed to determine significance.

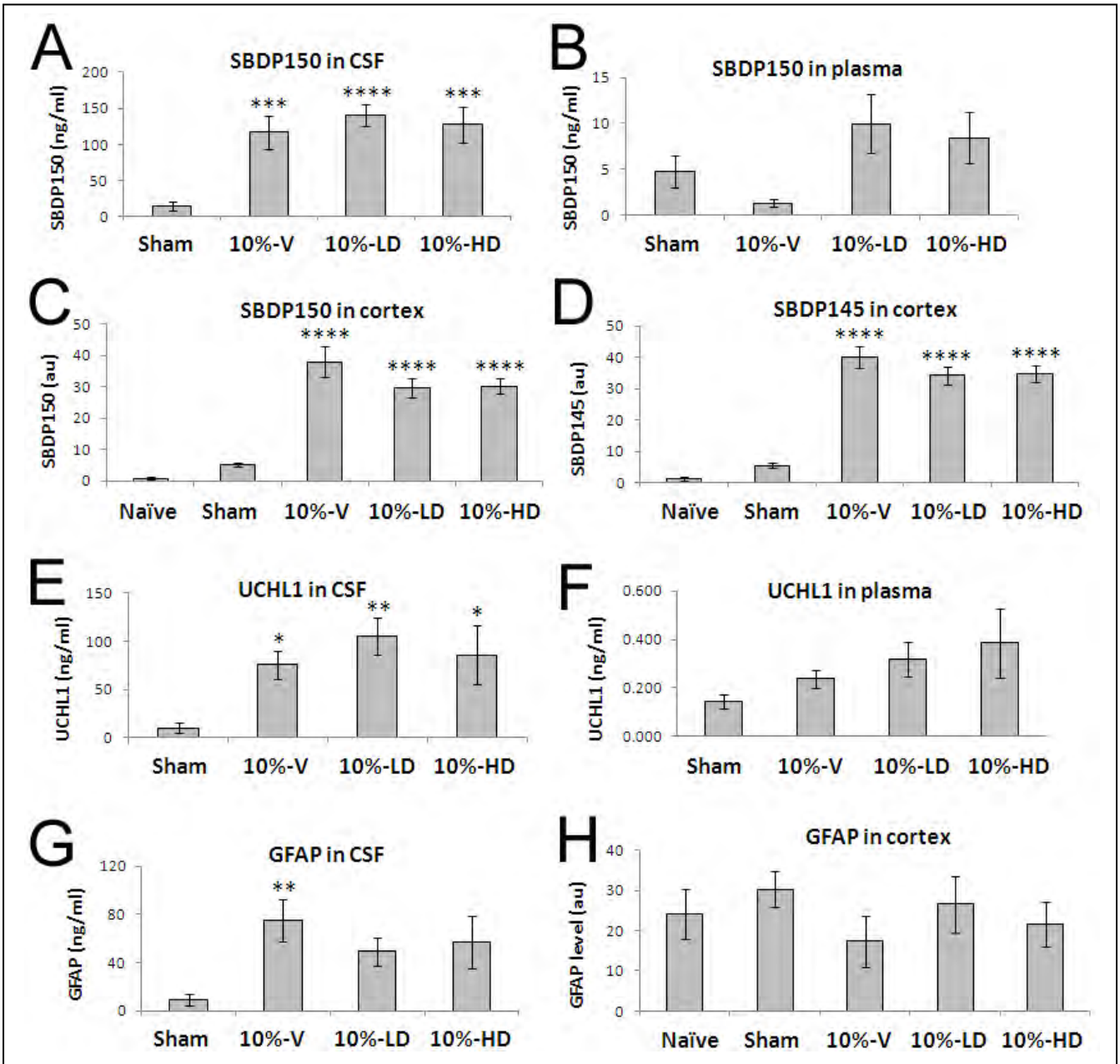



**Figure 14. Dextromethorphan decreased GFAP in CSF after PBBI but did not lower other biomarkers.**
**A-H.** All biomarker levels were measured in cortex and biofluids of rats at one day post 10% PBBI, or in uninjured controls (naïve, sham). PBBI animals were treated with vehicle (V) or DM at low (LD, 0.156 mg/kg) or high doses (HD, 10 mg/kg). All significant differences are indicated by asterix, and p values are relative to sham thorougout (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). **A.** SBDP150 levels were strongly elevated by 10% PBBI and not lowered by DM. **B.** SBDP150 levels in plasma did not differ among the groups. **C,D.** SBDP150 and SBDP145 levels were robustly increased by injury but not affected by DM. **E.** UCHL1 was increased by injury in CSF and not lowered by DM. **F.** UCHL1 in plasma did not change. **G.** GFAP levels were significantly elevated in CSF by injury (10%-V), but when DM was administered at either dose, GFAP levels no longer differed from sham. **H.** GFAP did not change in cortex.